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Running Head:

Metabolic coordination of starch degradation and crassulacean acid metabolism

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Leaf carbohydrates influence transcriptional and post-transcriptional regulation of nocturnal carboxylation and starch degradation in the facultative CAM plant, *Mesembryanthemum crystallinum*

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Abstract (263 words)

Nocturnal degradation of transitory starch is a limiting factor for the optimal function of crassulacean acid metabolism and must be coordinated with phosphoenolpyruvate carboxylase (PEPC)-mediated CO₂ uptake to optimise carbon gain over the diel cycle. The aim of this study was to test the hypothesis that nocturnal carboxylation is coordinated with starch degradation in CAM via a mechanism whereby the products of these pathways regulate diel transcript abundance and enzyme activities for both processes. To test this hypothesis, a starch and CAM-deficient mutant of *Mesembryanthemum crystallinum* was compared with wild type plants under well-watered and saline (CAM-inducing) conditions. Exposure to salinity increased the transcript abundance of genes required for nocturnal carboxylation, starch and sucrose degradation in both wild type and mutant, but the transcript abundance of several of these genes was not sustained over the dark period in the low-carbohydrate, CAM-deficient mutant. The diel pattern of transcript abundance for PEPC mirrored that of PEPC protein, as did the transcripts, protein, and activity of chloroplastic starch phosphorylase in both wild type and mutant, suggesting robust diel coordination of these metabolic processes. Activities of several amylase isoforms were low or lacking in the mutant, whilst the activity of a cytosolic isoform of starch phosphorylase was significantly elevated, indicating contrasting modes of metabolic regulation for the hydrolytic and phosphorylytic routes of starch degradation. Externally supplied sucrose resulted in an increase in nocturnal transcript abundance of genes required for nocturnal carboxylation and starch degradation. These results demonstrate that carbohydrates impact on transcriptional and post-transcriptional regulation of nocturnal carboxylation and starch degradation in CAM.

Introduction

In plants with crassulacean acid metabolism, a diel separation of carboxylation processes mediated by phosphoenolpyruvate carboxylase (PEPC) and ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) optimizes photosynthetic performance and carbon gain in water-limited environments. During CAM, malic acid is accumulated overnight via PEPC-mediated carboxylation and is subsequently broken down to release CO₂ that is fixed by RUBISCO during the following day behind closed stomata, thereby conserving water and resulting in water-use efficiencies that exceed those of C₄ and C₃ plants by at least 3- and 6-fold respectively (Borland *et al.*, 2009). The temporal separation of C₃ and C₄ carboxylation processes that defines CAM provides plasticity for optimizing carbon gain and water use in response to changing environmental conditions by extending or curtailing the period of net CO₂ uptake over a 24 h period (Dodd *et al.*, 2003). Thus, efficient operation of the pathway and the ecological success of CAM are based on mechanisms that synchronize the supply and demand for carbon whilst maintaining photosynthetic plasticity over the 24 h CAM cycle (Borland and Taybi, 2004).

Circadian control of carbon flux through PEPC is generally regarded as a key component underpinning the diel separation of carboxylation processes that define CAM (Nimmo, 2000). PEPC is activated at night via phosphorylation of a serine residue near the N-terminus of the protein that renders the enzyme more sensitive to PEP and the positive effectors, glucose-6-P and triose-P, and less sensitive to the allosteric inhibitor, malate (Nimmo *et al.*, 1986; Chollet *et al.*, 1996). Reversible phosphorylation of PEPC is catalysed by a dedicated Ser/Thr protein kinase (PEPc-kinase, PPCK), which in turn is regulated at the transcriptional level by the circadian clock (Hartwell *et al.*, 1999; Taybi *et al.*, 2000). Circadian control of the degradation of PPCK towards the end of the night might enable plants to anticipate dawn and ensure a rapid inactivation of PEPC at the start of the day,

thereby avoiding futile cycling of malate synthesis and decarboxylation. However, PEPC activation status can be modified by leaf metabolic status, so that *Ppck* transcripts can persist beyond the start of the photoperiod if cytosolic malate is maintained below a threshold concentration (Borland *et al.*, 1999). Because diel changes in malate content are a distinguishing feature of CAM, malate-elicited feedback inhibition of *Ppck* expression is thought to provide an effective means of fine-tuning CO₂ uptake over the day/night cycle in response to changes in environmental conditions (Borland and Taybi 2004). Thus, integration of circadian and environmental signals appears necessary for achieving the synchronization and plasticity of metabolism that is inherent to CAM.

PEPC-mediated carboxylation is intimately linked to the diel turnover of starch as the amount of CO₂ taken up during the night depends on the availability of C₃-carbon substrate (PEP) produced from the nocturnal degradation of starch accumulated during the day (Cushman *et al.*, 2008a, Ceusters *et al.*, 2014). The mechanisms and molecular targets for coordinating the reciprocal diel cycling of malate and carbohydrates that define CAM remain to be elucidated but, with analogy to the regulation of PEPC described above, starch degradation can be hypothesized to be subject to both circadian and metabolite control at transcriptional and posttranscriptional levels. In the C₃ model plant *Arabidopsis thaliana*, day/night and circadian oscillations of transcripts encoding enzymes of starch degradation have been reported (Harmer *et al.*, 2000; Schaffer *et al.*, 2001; Smith *et al.*, 2004) and it seems that the circadian clock plays a central role in coordinating day/night starch turnover with growth and export (Graf *et al.*, 2010; Graf and Smith, 2011). Posttranscriptional regulation has also been proposed to be important for timing starch breakdown in *A. thaliana* (Stitt and Zeeman, 2012), and the products of starch degradation have been implicated in sugar-mediated gene regulation of carbohydrate turnover, photosynthesis, and growth (Caspar *et al.*, 1985; Corbesier *et al.*, 1998, Stitt and Zeeman, 2012). For CAM, previous

work has shown that the induction of CAM in *Mesembryanthemum crystallinum* by exposure to salinity is accompanied by increased activities of a range of starch-degrading enzymes implicated in both the hydrolytic and phosphorylytic routes of starch degradation (Paul *et al.*, 1993). Moreover, robust circadian control of the transcript abundance of several genes implicated in carbohydrate turnover and chloroplast transport of C-metabolites has been reported to accompany CAM induction in *M. crystallinum* (Häusler *et al.*, 2000; Dodd *et al.*, 2003; Kore-eda *et al.*, 2005; Cushman *et al.*, 2008b). However, the influence of metabolite control on transcriptional and posttranscriptional regulation of starch degradation and its coordination with PEPC activation remains to be established. A further question relates to the levels of control exerted over the different genes and enzymes implicated in nocturnal starch degradation, given that C₃ and CAM plants appear to use different enzymatic routes to break starch down (Borland *et al.*, 2009; Weise *et al.*, 2011). Starch degradation is believed to be initiated by the concerted action of phosphoglucan water dikinase (PWD) and glucan water dikinase (GWD) disrupting the starch granules (Ritte *et al.*, 2002; Kotting *et al.*, 2005). In *A. thaliana*, the disrupted starch granules are then broken down *via* a hydrolytic route which is catalysed by α and/or β amylases (α AMY, β AMY) with maltose as the major product that is exported from the chloroplast (Weise *et al.*, 2005), although α AMY was shown to not be necessary for starch degradation in *A. thaliana* (Yu *et al.*, 2005). In the cytosol, maltose is converted to sucrose *via* the concerted action of disproportionating enzyme (DPE2) and a cytosolic α -glucan phosphorylase (STP2) Chia *et al.*, 2004; Lu *et al.*, 2006). In contrast, chloroplasts of CAM-performing *M. crystallinum* have been shown to predominantly export glucose-6-P (Neuhaus and Schulte, 1996; Kore-eda and Kanai, 1997), indicating that starch is degraded *via* the phosphorylytic route using chloroplastic α -glucan phosphorylase (STP1). Thus, in the CAM system, metabolite control of transcriptional and posttranscriptional

regulation of the phosphorylytic route of starch degradation and its coordination with PEPC activation might be predicted to differ from that of the hydrolytic route.

The aim of the work presented here was to test the hypothesis that the products of starch degradation coordinate and regulate nocturnal carboxylation and starch degradation at both transcriptional and posttranscriptional levels in the CAM system. To test this hypothesis, a starch and CAM-deficient mutant of *M. crystallinum* (Cushman *et al.*, 2008a) was compared with wild-type plants under well-watered and saline (CAM-inducing) conditions. Diel transcript abundances of genes implicated in PEPC activity and activation (*Ppc1*, *Ppck1*), the initiation of starch degradation (*Pwd1*, *Gwd1*), the hydrolytic route of starch degradation (α *Amy1*, β *Amy1*, *Dpe2*, *Stp2*), the phosphorylytic route of starch degradation (*Stp1*), and sucrose degradation (vacuolar invertase *V-Inv1*; sucrose synthase *Ssy1*) were compared in wild type and mutant under control and saline conditions. Diel patterns of protein abundance and activity were monitored in parallel for selected enzymes. Lastly, the transcript abundances of *Ppc1* and *Ppck1*, together with those of selected genes implicated in starch degradation were monitored over the day/night cycle in detached leaves fed with sucrose in order to directly assess the impact of sugars on the coordinated expression of these genes.

Material and Methods

Plant Material

Seeds of wild type and a starch-deficient *pgm* mutant (351) of the common ice plant (*Mesembryanthemum crystallinum*) (Cushman *et al.*, 2008a) were germinated in soil in a growth chamber under a 12-h light (26 °C)/12-h dark (18 °C) cycle. Ten-day-old seedlings were transplanted individually into 0.5 litre plastic pots containing soil (John Innes 2) and irrigated once daily with water. Plants were grown under fluorescent lighting of 400 μ mol .

$\text{m}^{-2} \cdot \text{s}^{-1}$ at plant height for the first 4 wk, then under $200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ to limit CAM expression, particularly in the mutant compared to wild type. Once per week, plants were watered with nutrient supplement (Phostrogen, UK). Four-week-old plants were subjected to salt treatment by watering daily for 10 d with about 50 ml of 400 mM NaCl solution per plant. The fourth pair of leaves was harvested from three plants of the wild type and the mutant every 4 h for 24 h, frozen in liquid- N_2 , and stored at -80°C . Sucrose feeding experiments were conducted on leaves detached from plants that had been treated with salt as above for 10 d. Fourth leaf pairs were excised, weighed, and placed in either water for the controls or in 0.25 M sucrose solution. Sugar treatments began after 3 h in the photoperiod and leaves were maintained in sugar solutions during the full duration of the experiment. Leaves were sampled every 4 h over 24 h beginning at 8 h of incubation in sucrose solution. Leaves were then frozen in liquid N_2 and stored at -80°C .

Extraction and measurement of carbohydrates and organic acids

Carbohydrates and organic acids were extracted by adding 1g of frozen, powdered plant material to 5 ml of 80% methanol and incubating at 80°C for 1 h. Titratable acidity was determined by titrating 1 ml of the methanol-soluble leaf extract with 1 mM NaOH to the pink phenolphthalein end point. Total soluble sugars were measured using the colorimetric phenol-sulphuric acid test of Dubois *et al.* (1956). For determination of starch content, the pellet remaining after methanol extraction was washed three times with 1 ml of 80 % methanol and three times with 1 ml of distilled water, and then resuspended by boiling for 30 min in 1 ml of acetate buffer (0.1 M Na acetate, 0.1 N acetic acid, pH 4.5). After cooling, starch was degraded into glucose residues by overnight incubation at 37°C in the presence of 3 U amyloglucosidase and 0.25 U α -amylase (Sigma-Aldrich, UK). The extract was then

centrifuged at 13,000 \times g and the supernatant used for sugar measurement using the phenol-sulphuric acid test.

RNA extraction and semi-quantitative RT-PCR conditions

Total RNA was isolated from leaves using the Tri-reagent method as previously described (Taybi and Cushman, 1999). Frozen, powdered leaf tissue (~200 mg) was extracted in 1 ml of Tri-reagent. Semi-quantitative RT-PCR assays for all of the genes analysed in the present study were conducted in a single-tube reaction as previously described (Taybi and Cushman, 1999), using 100 ng of DNase I-treated RNA for each reaction (see Table1 for primers). RT was conducted by holding at 50 °C for 30 min, followed by 25 PCR cycles consisting of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. The polyubiquitin gene (BE036552) was used to control for equal RNA input and similar RT-PCR conditions and used to normalize the transcript level data.

Extraction and in-gel activity measurements of amylases, α -glucan phosphorylases, and disproportionating enzyme (DPE2)

Activities of amylases and chloroplastic and cytosolic isoforms of α -glucan phosphorylase were monitored in leaf extracts prepared by homogenizing 200 mg of frozen powdered leaf tissue in 1 ml of extraction buffer containing 0.3 M HEPES pH 7.4, 20 mM MgCl₂, 1 mM EDTA, 0.5 % PVP (w/v), 2 mM DTT and 4 mM benzamidine. Extracts were clarified by centrifugation at 13,000 \times g for 10 min at 4 °C. Soluble protein content in the extracts was determined using the Bradford reagent (Bradford, 1976). Protein extracts were resolved on native PAGE, based on a modified method from Zeeman *et al.* (1998). Discontinuous native polyacrylamide gels contained 0.1% (w/v) amylopectin (Sigma, Dorset, UK) for amylase activities and 0.8% (w/v) glycogen (Sigma, Dorset, UK) for starch

phosphorylase activities. Following electrophoresis of 40 µg of total protein extract per sample, the gels for amylase activities were incubated overnight at 37 °C in 100 mM sodium-acetate buffer pH 6.0 containing 1mM MgCl₂, 1 mM CaCl₂ and 1 mM DTT. Degradation of amylopectin was visualised by contrast as white bands after staining the gel with iodine. The activities of cytosolic and chloroplastic isoforms of α-glucan phosphorylase (STP) were measured in the starch biosynthetic direction after an overnight incubation of the gels in 0.1 M Na-Citrate pH 6.5 containing 0.05% (w/v) soluble starch and 20 mM glucose-1-phosphate at room temperature (Malinova *et al.*, 2014). Chain extension of starch was visualised by iodine staining and appeared as blue bands. For detection of disproportionating enzyme (DPE2) activity, the gels were treated as for STP except that glucose-1-phosphate was replaced by 30 mM maltose (Malinova *et al.*, 2014).

Immunoblots for PEPc, GWD, STP and DPE2

Exactly 25 µg of protein extracts for PEPc and GWD and 40 µg for STP per sample were separated on 12% denaturing polyacrylamide gels, blotted onto polyvinylidene fluoride membranes (Sigma, Dorset, UK). Polyclonal anti-PEPc (*M. crystallinum*), anti-GWD (*Arabidopsis*), and monoclonal anti-STP (J3b, sweet potato, Chen *et al.*, 2002) or anti-DPE2 antibodies were then used for immunodetection of the respective bands. The chemiluminescent detection system ECL (Amersham, Buckinghamshire, UK) was used to visualise the bands following manufacturer's instructions.

Sampling and statistical analysis

Three samples were taken every four hours over a day/night cycle. Each sample consisted of two leaves (from pair four counting from the bottom of the plant) taken from two different plants and ground together. Measurements of organic acids, sugars, transcript,

protein and enzyme activity levels, were done in each replicate. Quantification of the bands from the gel pictures of RT-PCR and STP-activity gels was done twice for each gel and averaged. The quantification data were then averaged from the three samples and the standard errors calculated at 95% confidence. Normality of the distribution of the data was verified in Excel and an independent sample t-test was calculated for the means of key time points (10:00 for titratable acidity, 18:00 for sugars and 02:00 for STP activities) to determine the p significance values between the wild type and the mutant. Differences between the wild-type and the mutant were considered to be significant only when the p-values were <0.05.

Results

CAM expression and day/night patterns of carbohydrate turnover

To assess the effect of salt treatment on CAM expression and carbohydrate status in the wild type and the *pgm* mutant of *M. crystallinum*, changes in leaf titratable acidity (TA) were monitored over a 24 h cycle in leaves of control plants and plants subjected to salt treatment (0.4 M NaCl for 10 d). Salt treatment resulted in a fourfold increase in the diel turnover of titratable acids in leaves of wild type plants, indicating the induction of CAM (Fig. 1A). Under salinity treatment, the *pgm* mutant showed low nocturnal accumulation of TA comparable to that in the control wild type and two and half fold lower than that in salt-treated wild type, indicating a substantial reduction in CAM activity in this mutant ($p < 0.05$) (Fig 1B).

Soluble sugar contents were more than twofold higher in leaves of wild-type control plants compared to the mutant (Figs. 1C and 1D). Salt treatment resulted in a substantial depletion of soluble sugars in both wild type and mutant, resulting in comparable sugar contents in leaves of wild type and mutant ($p < 0.05$) (Figs. 1C and 1D).

Salinity treatment elicited a significant (over threefold) depletion of leaf starch content in wild-type plants, although the amount of starch degraded overnight was comparable in control and salt-treated plants (Fig. 1E). Overall, salt treatment elicited a shift away from soluble sugars toward starch dominating diel carbohydrate turnover in wild-type plants. As expected, levels of starch in the mutant were extremely low in both the control and salt-treated plants (Fig. 1F) and were fifty times lower than the wild-type ($p < 0.05$).

Transcript abundances of *Ppc1* and *Ppck1* and genes implicated in starch and sucrose degradation in leaves of wild type and *pgm* mutant

Salt treatment resulted in a strong increase in transcript abundance of both *Ppc1* and *Ppck1* genes in wild type (Fig. 2A and Fig. S1) and the *pgm* mutant (Fig. 2B and Fig. S1). *Ppc1* transcripts showed a distinct oscillation over the day/night cycle in both salt-treated wild-type and mutant plants, although the timing of transcript decay occurred earlier in the dark in the mutant compared to wild type. Transcript abundances for genes implicated in starch and sucrose degradation were also monitored to assess any possible coordination of expression with the genes required for nocturnal carboxylation (*i.e.*, *Ppc1*, *Ppck1*) and to examine if the contrasting diel turnover of metabolites found in wild type and the *pgm* mutant were reflected in altered diel patterns of gene expression. The transcript abundances of nine genes encoding enzymes implicated in starch and sucrose degradation were monitored over the 24 h light/dark cycle. These genes included α -amylase 1 (*α Amy1*), β -amylase 1 (*β Amy1*), cytosolic and plastidic starch phosphorylases (*Stp1*, *Stp2*), glucanotransferase/disproportionating enzyme (*Dpe2*), water glucan dikinase (*Gwd1*), phosphoglucan dikinase (*Pwd1*), vacuolar invertase (*V-Inv1*), and sucrose synthase (*Ssy1*). A marked day/night oscillation of transcript abundances of all genes was apparent in both the control plants and plants subjected to salt treatment (Fig. 2). Under salt treatment, transcript

abundances increased and transcripts persisted for longer over the day/night cycle compared to those in control plants for seven of the nine genes (i.e. α *Amy*, β *Amy1*, *Stp1*, *Stp2*, *Dpe2*, *V-Invl*, *Ssy1*) in both wild type and the mutant (Fig. 2 and Figs. S2, S3 and S5). However, in general, the transcript abundances of these seven genes were not sustained over the dark period in the salt-treated starch/CAM-deficient mutant as compared to wild type, suggesting an impact of metabolites on transcript persistence (Fig. 2). Salt treatment resulted in a decrease in *Gwd1* transcript abundance in both the wild type and the mutant (Figs. 2A and 2B). However, transcript amounts for *Pwd1* remained unaffected by salt treatment in both the wild type and the mutant. In terms of the diel timing of transcript abundance, in salt-treated wild type plants (i.e., strong CAM-performing plants), α *Amy1*, β *Amy1*, *Stp1*, and *Stp2* transcripts were at greatest abundance from the middle of the photoperiod to the middle of the dark period (i.e., from 14:00 to 02:00; Fig. 2). Despite the contrasting diel changes in malic acid and carbohydrates noted between the wild type and mutant, the pattern of diel transcript abundance for *Stp1* closely mirrored that of *Ppc1* in salt-treated wild type and mutant, suggesting robust diel coordination of these genes. *Gwd1* and *Pwd1* transcripts, which encode regulatory kinases that initiate starch degradation, showed reciprocal patterns of expression that were also distinct from the diel expression patterns noted for amylase and starch phosphorylase genes. *Dpe2* transcripts, which encode a glycosyltransferase involved in cytosolic maltose degradation, showed very different diel patterns of transcript abundance between control and salt-treated plants and between wild type and mutant (Figs. 2A and 2B). In control wild-type plants, *Dpe2* transcripts were most abundant over the second half of the dark period, but in salt-treated CAM-performing wild-type plants, transcripts were abundant throughout the diel cycle. In control mutant plants, *Dpe2* transcripts were most abundant at the end of the photoperiod, but exposure to salinity shifted the peak of transcript abundance to the start of the dark period (Fig. 2 and Fig. S3). Such results indicate a profound impact of

metabolite content on transcriptional regulation of *Dpe2*. The diel timing of transcript abundance of the sucrose-degrading genes *V-Inv1* and *Ssy1* did not mirror those of the genes encoding enzymes for starch degradation or C₄ carboxylation (Fig. 2 and Fig. S5).

Activities of starch- and maltose-degrading enzymes: amylases (AMY), α -glucan (starch) phosphorylase (STP), and glucanotransferase (DPE2)

To examine whether activities of hydrolytic enzymes, *i.e.*, α -amylases (hydrolysis of α -1,4-linkage, EC 3.2.1.1) and β -amylases (hydrolysis of 1,4 and 1,6 linkages, EC 3.2.1.2), starch phosphorylases (α -1,4-glucan phosphorylase, EC 2.4.1.1), and glucanotransferase/disproportionating enzyme (DPE2) followed the same pattern as changes in their transcript abundances, native PAGE was performed using leaf extracts prepared from control plants and plants subjected to 10 d of salinity treatment. Wild-type plants contained more isoforms of hydrolytic enzymes compared to the mutant (Fig. 3). A strong increase in amylase activities and the appearance of new isoforms was induced by salinity in both wild type and mutant. At least 6 different isoforms were detected in salted wild type and 4 in the mutant (Fig. 3). One amylase isoform showed a much higher activity compared to the others and this isoform was present in higher abundance in the mutant (Fig. 3, asterisk). There was no apparent change in hydrolytic enzyme activities over the day/night cycle suggesting that diel changes in transcript abundances (Figs. 2A and 2B) were not reflected by changes in protein abundance.

The activities of cytosolic and plastidic isoforms of STP increased under salinity treatment in both wild type and mutant (Fig. 4). In contrast to the hydrolytic enzymes under salt treatment, the diel changes in activities of cytosolic and plastidic isoforms of STP in the wild-type plants reflected the diel change in transcript abundances for both *Stp1* and *Stp2*, with highest activities noted early in the dark period (Fig. 4). In the mutant, the activity of

cytosolic STP was substantially higher than that in the wild type and under salinity, the enzyme activities of both cytosolic and plastidic isoforms stayed high longer during the night compared to those in wild type (at 02:00, $p < 0.05$).

The activity of glucanotransferase/disproportionating enzyme (DPE2) over the day/night cycle in control and salt-treated plants was also compared in wild-type and mutant plants (Fig. 5). While salinity resulted in a strong increase in the activity of DPE2 in the wild type plants, this was not observed in the mutant, where DPE2 activity was below the limits of detection (Fig. 5).

Protein contents of PEPC, glucan water dikinase, starch phosphorylase, and glucanotransferase

To determine if the diel and salt-induced changes in transcript abundances (Figs. 2A and 2B) resulted in changes in protein amounts, protein gel blot analysis was performed to assess the protein content of PEPC, GWD, STP and DPE2. A strong increase in the abundance of PEPC protein was noted in salt-treated, CAM-performing wild-type plants (Fig. 6). Moreover, a diel change in PEPC protein abundance was noted in salt-treated plants, which mirrored the diel change in *Ppc1* transcripts (Fig. 2), suggesting rapid turnover of the protein. Salt elicited a decrease in GWD protein (Fig. 6), which is consistent with the salt-induced decline in *Gwd1* transcripts (Fig. 2). Moreover, a diel change in GWD protein abundance was observed in salt-treated wild-type plants. The protein abundances of STP and DPE2 also increased under salinity (Fig. 6), which correlated with salt-induced increases in transcript abundances (Fig. 2) and enzyme activities (Figs. 4 and 5).

Impact of sucrose feeding on diel transcript abundance of *Ppc1*, *Ppck1*, *Gwd1*, *Stp2*, and α *Amy1*

To examine the potential impact of carbohydrate status on the transcription of genes required for C₄ carboxylation and starch degradation, detached leaves from wild-type plants induced to CAM were incubated with the petiole in a sucrose solution (see Material and Methods for treatment time and conditions) to maintain high levels of sugars in the leaf cells. Preliminary data indicated that detached leaves were capable of metabolizing exogenous sucrose, as an increase in glucose and fructose content in leaves incubated in sucrose has been noted (E. Antony, A. Borland and T. Taybi, unpublished data). There was substantial uptake of sucrose by the leaves and soluble sugar content increased by over 80-fold compared to the control leaves maintained with the petiole in water (Fig. 7B). There was no significant difference in sugar content between leaves fed with sucrose for 8 h (18:00) and those incubated in sucrose for 24 h (10:00). Significant nocturnal acidification was observed in detached leaves maintained under both water (control) and sucrose treatment (Fig. 7A), but there was no significant increase in nocturnal acidification as a consequence of sugar feeding. The diel pattern of *Ppc1* transcript abundance in detached control leaves was similar to that *in planta* (cf. Fig. 7C and Fig. 2). Sucrose feeding resulted in an increase in the abundance of *Ppc1* transcripts and caused a shift in the day/night pattern of transcript accumulation, with *Ppc1* transcript abundance remaining high for the entire night in the sucrose-fed leaves. *Ppck1* transcripts showed similar diel patterns of abundance in the control and the sucrose-fed leaves, although, as with *Ppc1*, the nocturnal abundance of *Ppck1* transcripts was higher in sucrose-fed leaves. Similarly, sucrose feeding resulted in an increase in nocturnal transcript abundance for both *Gwd1* and *Stp2* genes (Fig. 7C). In contrast, sugar feeding did not significantly change the transcript abundance of α *Amy1*.

Discussion

The aim of this study was to test the hypothesis that in CAM, nocturnal carboxylation is coordinated with starch degradation *via* a mechanism whereby the products of these pathways (*i.e.*, malic acid, sugars) regulate the diel transcript abundance and enzyme activities required for both processes. To test this hypothesis, wild-type plants of *M. crystallinum* were compared with mutant plants deficient in chloroplastic phosphoglucomutase (PGM) under well-watered (control) or saline (0.4 M NaCl for 10 d) conditions. The *pgm* mutant was shown to be deficient in starch and exhibited suppressed diel cycling of malic acid and sugars under saline conditions, as reported previously (Cushman *et al.*, 2008a; Haider *et al.*, 2012). Thus, by comparison with the wild type, the CAM- and starch-deficient *pgm* mutant of *M. crystallinum* provides a means of examining the impact of the reciprocal day/night cycling of malic acid and carbohydrates on the diel expression and coordination of enzymes necessary for nocturnal carboxylation.

Impact of metabolites on the transcriptional regulation and coordination of C₄ carboxylation and starch degradation

Posttranslational modification of PEPC, which is mediated by a dedicated protein kinase (PPCK), is considered a major point of control over the nocturnal activation of C₄ carboxylation in CAM plants (Nimmo *et al.*, 2000). PEPC phosphorylation is positively correlated with the abundance of *Ppck* transcripts and results in PEPC activation at night (Hartwell *et al.*, 1999). The data presented here confirm previous observations that *Ppck1* transcripts accumulate progressively during the night, reaching a maximum towards the end of the night, and decline during the day. However, our observation that diel changes in *Ppck1* transcript abundance are paralleled by diel changes in PEPC protein also indicate that diel transcriptional control over PEPC protein abundance occurs in salt-treated plants of *M.*

crystallinum. This suggests rapid turnover of PEPC protein and that consequent changes in abundance of PEPC protein could provide an additional layer of control over C₄ carboxylation. Our data suggest reciprocal interplay between transcriptional regulation of PEPC and PPCK, because *Ppc1* transcripts begin to decline when *Ppck1* transcript accumulation is at its maximum during the night, and *Ppc1* transcripts increase during the day when *Ppck1* transcripts were at a minimal level. Despite the early decline in PEPC transcripts and protein towards the end of the night, titratable acids continued to accumulate right through to the start of the photoperiod, which may be attributed to the persistence of PPCK in maintaining PEPC activity despite a reduced enzyme quantity at the end of the night. The transcriptional regulation of PEPC and PPCK also seems to be subject to metabolite control given the reduced abundance of *Ppc1* and *Ppck1* transcripts in the CAM and starch-deficient *pgm* mutant. Indeed, sugar feeding results in increased nocturnal abundance of *Ppc1* and *Ppck1*, indicating a further layer of control over nocturnal carboxylation that could serve to maximise dark CO₂ uptake when substrate is in plentiful supply. This observation support the importance of the regulation of CAM by carbohydrates as reported earlier by Ceusters *et al.* (2009), using carbon budgets, these authors have shown that CAM operation under drought was dependent upon the supply of PEP which originated flexibly from starch or sucrose depending on stress. Salinity mediates an increase in the transcript abundance of genes implicated in starch and sucrose degradation in both wild type and mutant. Distinct diel oscillations in transcript abundance noted for most of these genes have also been reported for *Arabidopsis* and *M. crystallinum* (Smith *et al.*, 2004; Cushman *et al.*, 2008b). Overall the diel oscillations in transcript abundance for starch-degradation genes were consistent with what was previously reported (Dodd *et al.*, 2003; Cushman *et al.*, 2008b). Under salinity, genes such as α *Amy1*, β *Amy1*, *Stp1*, and *Stp2* show an increase in transcript abundance during the day and a decline during the latter part of the dark period,

consistent with the patterns observed for these genes in *A. thaliana*. Close transcriptional coordination of the abundance of *Ppc1* and *Stp1* in particular was observed over the diel cycle in both salt-treated wild type and mutant. Moreover, the diel cycling of *Stp1* transcripts was mirrored by a diel change in the protein abundance of STP and activity of chloroplastic starch phosphorylase (glucan hydrolase) in both salt-treated wild type and *pgm* mutant. Such observations suggest coordination of nocturnal PEPC activity and starch degradation by the phosphorylytic route *via* robust diel/circadian control of gene transcription independent of reciprocal cycling of malic acid and starch.

The salt-induced changes in gene expression observed for *M. crystallinum* might have arisen as an indirect response to carbohydrate limitation or even C-starvation caused by salinity. For example, in citrus roots, carbohydrate starvation results in a strong increase in α -amylase transcript abundance, but a strong decrease in transcript abundance for starch phosphorylases has been observed (Li *et al.*, 2003). Our sugar feeding experiments actually indicated an increase in transcript abundance of some genes implicated in starch degradation (i.e. *Gwd1* and *Stp2*), while the abundance of α -*Amy1* transcripts was unchanged. Thus, it seems unlikely that the salt-induced increase in transcript abundances of genes implicated in starch and sugar degradation in *M. crystallinum* resulted from carbohydrate depletion. Metabolite content did however influence the diel persistence of several genes implicated in starch and sucrose degradation (e.g., α *Amy1*, β *Amy1*, *Stp1*, *Dpe2*, *V-inv1*, *Ssy1*), as observed by the reduced nocturnal transcript abundances in the low-carbohydrate/CAM-deficient mutant. Such data indicate a role for metabolites in modulating transcriptional regulation of starch and sucrose degradation to match substrate availability.

Different enzymatic steps and routes for starch degradation are subject to contrasting layers of regulation in *M. crystallinum*

In *A. thaliana*, the large diel changes in transcript levels observed for many starch-degrading genes do not, in general lead to significant changes in the levels of the encoded proteins (Smith *et al.*, 2004; Lu *et al.*, 2005). Because it can take several days for a change in transcription to lead to a substantial change in the amount of the encoded protein, diel changes in transcription have been proposed to serve to adjust starch turnover to mid-term and long-term conditions (Stitt and Zeeman, 2012). In contrast and as described above, in *M. crystallinum*, diel transcript abundances for *Ppc1* and *Stp1* were mirrored by diel changes in the relevant protein and enzyme activities. A similar mirroring of transcript abundance and protein content for glucan water dikinase (GWD), which is believed to initiate the process of starch degradation, was also noted in *M. crystallinum*. In *Arabidopsis* GWD proteins levels remain unchanged over the day/night cycle despite large changes in transcript levels (Skeffington *et al.*, 2004). Thus, diel transcriptional control of key starch degradative enzymes might be postulated as a functional point of divergence between C₃ and CAM plants.

For other enzymes, such as α *Amy1*, β *Amy1*, and *Dpe2*, which are implicated in the hydrolytic route of starch degradation and the processing of maltose exported from the chloroplast, salt-induced up-regulation of transcript abundance translated into a strong increase in amylase and DPE2 activities, but with no discernible day/night change in enzyme activities. Such observations indicate that other posttranscriptional mechanisms might be responsible for adjusting the rate of starch degradation that is catalysed *via* the hydrolytic route as opposed to the phosphorylytic route, which is believed to be the predominant route for starch degradation in CAM plants (Häusler *et al.*, 2000; Weise *et al.*, 2011).

Metabolic status appears to play a key role in modulating transcriptional and post-transcriptional regulation of starch degradation in *M. crystallinum*. The transcript and protein abundance of GWD showed a significant decrease in wild-type plants under salinity

treatment, which parallels the decline in starch and soluble sugars under salinity. Because sucrose feeding resulted in an increase in transcript abundance of *Gwd1*, *M. crystallinum* might be able to adjust the amount of GWD protein to align with leaf starch content *via* a sugar-signalling mechanism. The *pgm* mutant was found to be deficient in a number of amylase activities and in DPE2 activity when compared to the wild type. In contrast, starch phosphorylase activities, particularly those of the cytosolic isoform, show elevated activities in the mutant. Taken together, the data show that metabolites can influence starch degradation at both transcriptional and posttranscriptional processes. Moreover, enzymes that catalyse potentially different routes of starch degradation (*i.e.*, the hydrolytic and phosphorylytic routes) are subject to contrasting layers of control. Thus, the different layers of regulation over starch degradation reported here might serve to accommodate the well-documented plasticity of CAM expression in response to changes in environmental conditions. In summary, these results show that changes in carbohydrate levels over the day/night cycle might influence the level of expression of C₄ carboxylation and that the regulation of genes involved in starch or sucrose metabolism might influence the amplitude, and potentially the day/night pattern of CAM expression.

Conclusions

From the obtained results the following main conclusions can be drawn:

- Phosphoenolpyruvate carboxylase oscillates at the transcript and protein levels over the day/night cycle in *M. crystallinum*. In addition to the day/night regulation of the enzyme, these oscillations provide additional plasticity in terms of CO₂ assimilation via the CAM pathway.

- Transitory starch is required for CAM operation in *M. crystallinum*; lack of starch synthesis results in no CAM activity with perturbed patterns of starch degrading gene transcripts and enzymes.
- Salt-stress results in an increase in the transcript levels and activities of starch degrading enzymes and a changed day/night patterns of some starch degrading genes and enzymes.
- Starch-degradation during CAM expression in *M. crystallinum* uses the phosphorylytic route of starch degradation and involves regulation at the transcript and protein levels.
- Carbohydrates coordinate the regulation of C4-carboxylation and starch degradation in *M. crystallinum*.

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Figure legends

Figure 1. Titratable acidity, soluble sugars, and starch contents measured over the 24-h cycle in the wild type and a starch-deficient mutant of *Mesembryanthemum crystallinum* under day/night conditions in control plants (H₂O) and plants treated with NaCl (0.4 M) for 10 d. Each point is the mean of three replicates \pm standard error. The shaded boxes indicate the dark period.

Figure 2. Steady-state transcript levels monitored by RT-PCR for genes encoding the CAM isoform of phosphoenolpyruvate carboxylase (*Ppc1*); PEPc-kinase (*Ppck1*); genes encoding enzymes for starch degradation including amylases (α *Amy1*, β *Amy1*), starch phosphorylases (*Stp1*, *Stp2*), glucanotransferase (*Dep2*), glucan water dikinase (*Gwd1*), and phosphoglucan water dikinase (*Pwd1*); and genes implicated in sucrose degradation including vacuolar invertase (*V-Inv*) and sucrose synthase (*Ssy1*) over the day/night cycle in wild-type (A) and a starch- and CAM-deficient mutant (Cushman *et al.*, 2008) (B) of *Mesembryanthemum crystallinum* leaves from control plants or plants subjected to 10 d of salt treatment (0.4 M NaCl). Ubiquitin gene (*Ubq1*) transcripts were assayed as a control for equal RNA input and consistent RT-PCR conditions.

Figure 3. In-gel amylase activities over the day/night cycle in leaves from wild-type or starch-deficient mutant plants of *Mesembryanthemum crystallinum* under control conditions and under salt treatment (10 d, 0.4 M NaCl). Arrows indicate amylase activities, and asterisks indicate the strongest amylase activity which is higher in the starch-deficient mutant. Amylase activities were measured three times using samples from three biological replicates, results of a typical replicate are shown.

Figure 4. In-gel activities of cytosolic and chloroplastic starch phosphorylase over the day/night cycle in the wild type or starch-deficient mutant of *Mesembryanthemum crystallinum* under control conditions and under salt treatment (10 d, 0.4 M NaCl). Starch phosphorylase activities were measured three times using samples from three biological replicates, results of a typical replicate are shown.

Figure 5. In-gel glucanotransferase (DPE2) activities over the day/night cycle in the wild type or starch-deficient mutant of *Mesembryanthemum crystallinum* under control conditions and under salt treatment (10 d, 0.4 M NaCl). DPE2 activities were measured three times using samples from three biological replicates, results of a typical replicate are shown

Figure 6. Immunoblots for phosphoenolpyruvate carboxylase (PEPc), glucan water dikinase (GWD), starch phosphorylase (STP), and glucanotransferase (DPE2) over the day/night cycle in *Mesembryanthemum crystallinum* plants under control conditions and after 10 d of salt treatment (0.4 M NaCl). The immunoblots were performed three times using samples from three biological replicates, an immunoblot of typical replicate is shown.

Figure 7. Titratable acidity (A), soluble sugar content (B), and transcript levels for *Ppc1*, *Ppck1*, *Gwd1*, *Stp2*, and α *Amy1* genes (C) in *Mesembryanthemum crystallinum* leaves detached from plants that were subjected to one week of salt treatment (0.4 M NaCl) and incubated with the petiole in 250 mM sucrose solution or water (Control) (See Materials and Methods). *Ubq1* gene transcript levels were used as a control as above. Standard errors were calculated from three biological replicates.

Supplementary data

Supplementary figure S1. Relative steady state transcript levels monitored by RT-PCR for C4-carboxylation genes: genes encoding the CAM isoform of phosphoenolpyruvate carboxylase (*Ppc1*), and PEPc-kinase gene (*Ppck1*), in leaves from control plants and plants subjected to 10 days of salt-treatment (0.4 M NaCl) over the day/night cycle in leaves of wild-type and a starch- and CAM-deficient mutant of *Mesembryanthemum crystallinum*. Transcripts for ubiquitin gene (*Ubq1*) were used as control for equal RNA input and same RT-PCR conditions. The relative transcript abundances were measured via band quantification using the UV-tech software (UVtech, Cambridge, UK) and normalised to *Ubq1*. The shaded box indicates the dark period. The data are mean values of three biological replicates and bars represent standard errors.

Supplementary figure S2. Relative steady state transcript levels monitored by RT-PCR starch degradation genes, amylases (α *Amy1*, β *Amy1*), in leaves from control plants and plants subjected to 10 days of salt-treatment (0.4 M NaCl) over the day/night cycle in leaves of wild-type, and a starch- and CAM-deficient mutant of *Mesembryanthemum crystallinum*. Transcripts for ubiquitin gene (*Ubq1*) were used as control for equal RNA input and same RT-PCR conditions. The relative transcript abundances were measured via band quantification using the UV-tech software (UVtech, Cambridge, UK) and normalised to *Ubq1*. The shaded box indicates the dark period. The data are mean values of three biological replicates and bars represent standard errors.

Supplementary figure S3. Relative steady state transcript levels monitored by RT-PCR starch degradation genes including starch phosphorylases (*Stp1*, *Stp2*), glucanotransferase (*Dep2*), in leaves from control plants and plants subjected to 10 days of salt-treatment (0.4 M NaCl) over the day/night cycle in leaves of wild-type and a starch- and CAM-deficient mutant of *Mesembryanthemum crystallinum*. Transcripts for ubiquitin gene (*Ubq1*) were used as control for equal RNA input and same RT-PCR conditions. The relative transcript abundances were measured via band quantification using the UV-tech software (UVtech, Cambridge, UK) and normalised to *Ubq1*. The shaded box indicates the dark period. The data are mean values of three biological replicates and bars represent standard errors.

Supplementary figure S4. Relative steady state transcript levels monitored by RT-PCR for the genes involved in the regulation of starch degradation genes including glucan water dikinase (*Gwd1*) and phosphoglucan water dikinase (*Pwd1*) in leaves from control plants and plants subjected to 10 days of salt-treatment (0.4 M NaCl) over the day/night cycle in leaves of wild-type and a starch- and CAM-deficient *Mesembryanthemum crystallinum*. Transcripts for ubiquitin gene (*Ubq1*) were used as control for equal RNA input and same RT-PCR conditions. The relative transcript abundances were measured via band quantification using the UV-tech software (UVtech, Cambridge, UK) and normalised to *Ubq1*. The shaded box indicates the dark period. The data are mean values of tree biological replicates and bars represent standard errors.

Supplementary figure S5. Relative steady state transcript levels monitored by RT-PCR for genes implicated in sucrose degradation including vacuolar invertase (*V-Inv*) and sucrose synthase (*Ssy1*) in leaves from control plants and plants subjected to 10 days of salt-treatment (0.4 M NaCl) over the day/night cycle in leaves of wild-type and a starch- and CAM-deficient mutant of *Mesembryanthemum crystallinum*. Transcripts for ubiquitin gene (*Ubq1*) were used as control for equal RNA input and same RT-PCR conditions. The relative

transcript abundances were measured via band quantification using the UV-tech software (UVtech, Cambridge, UK) and normalised to *Ubp1*. The shaded box indicates the dark period. The data are mean values of average measurements of three biological replicates and bars represent standard errors.

Table 1: Genes and proteins, GenBank accession number (ACC), primer sets used in RT-PCR, and size of PCR products.

Gene	Enzyme/Protein	ACC	Primer sequence	RT-PCR product
<i>Ppc1</i>	Phosphoenolpyruvate carboxylase	X13660	Forward: 5'-CACTTAAACATGTCCTTGAG-3' Reverse: 5'-TAGCTTCACCAGCTCAGC-3'	419 bp
<i>Ppck1</i>	Phosphoenolpyruvate carboxylase kinase	AF158091	Forward: 5'-TTCGGAGCAGGAGAACAG-3' Reverse: 5'-ACTGGTTACCCAAGGATG-3'	340 bp
<i>αAmy1</i>	Alpha Amylase	BE130895	Forward: 5'-ACTCCAATGGCAAGCCAAGT-3' Reverse: 5'-GAAACTGAATAAGCATAGAGC-3'	531 bp
<i>βAmy1</i>	Beta amylase	AA962939	Forward: 5'-CTTGCACGGTATGATAGC-3' Reverse: 5'-CATTGGTTTGGTTGCTTCC-3'	297 bp
<i>Stp1</i>	Cytosolic starch phosphorylase	AI026304	Forward: 5'-GAAGTGAGCTATCACAG-3' Reverse: 5'-TTGTCCTGGTATGCTTCG-3'	409 bp
<i>Stp2</i>	Plastidic starch phosphorylase	CA837841	Forward: 5'-TGTGCTGATTGGGACTCTTG-3' Reverse: 5'-AGCCTCTTATTGGTGCAGGA-3'	700 bp
<i>Dpe2</i>	Disproportionating enzyme	AI823034	Forward: 5'-GAGCAGTGGATCTGTATG-3' Reverse: 5'-GCCTCTGAATCCAACAAGCTA-3'	604 bp
<i>Gwd1</i>	Water glucan dikinase	CA838032	Forward: 5'-TGTGCTCGACTATTCAAC-3' Reverse: 5'-TAATCAGTAGAGAACATCGAC-3'	637 bp
<i>Pwd1</i>	Phosphoglucan water dikinase	CA834228	Forward: 5'-CAGGTTTCCTCAATGCACT-3' Reverse: 5'-CAGGAACAATGCTATCAAC-3'	540 bp
<i>V-Inv1</i>	Vacuolar invertase	AY750150	Forward: 5'-CCTATTCAGCCGTGTGG-3' Reverse: 5'-AACCGGAAGTATCCAACC-3'	537 bp
<i>Ssy1</i>	Sucrose synthase	AA720478	Forward: 5'-CTGGAAGTACGTCTCTAAC-3' Forward: 5'-CTGGAAGTACGTCTCTAAC-3'	303 bp
<i>Ubp1</i>	Polyubiquitin	BE036552	Forward: 5'-CTTGCTGACTACAACATCC-3' Reverse: 5'-GAACCCTCCTTGTCTGG-3'	600 bp

Figure 1.

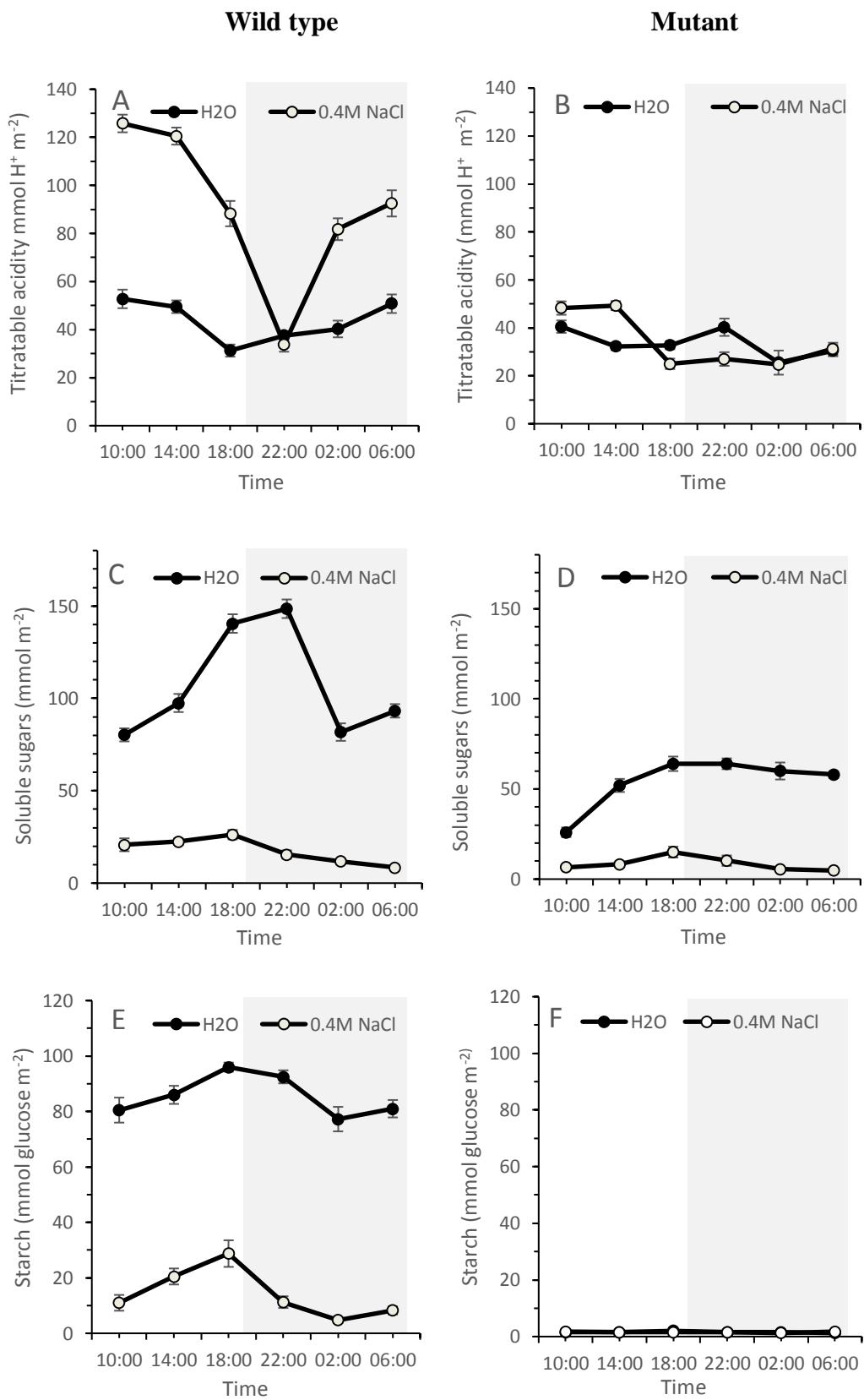
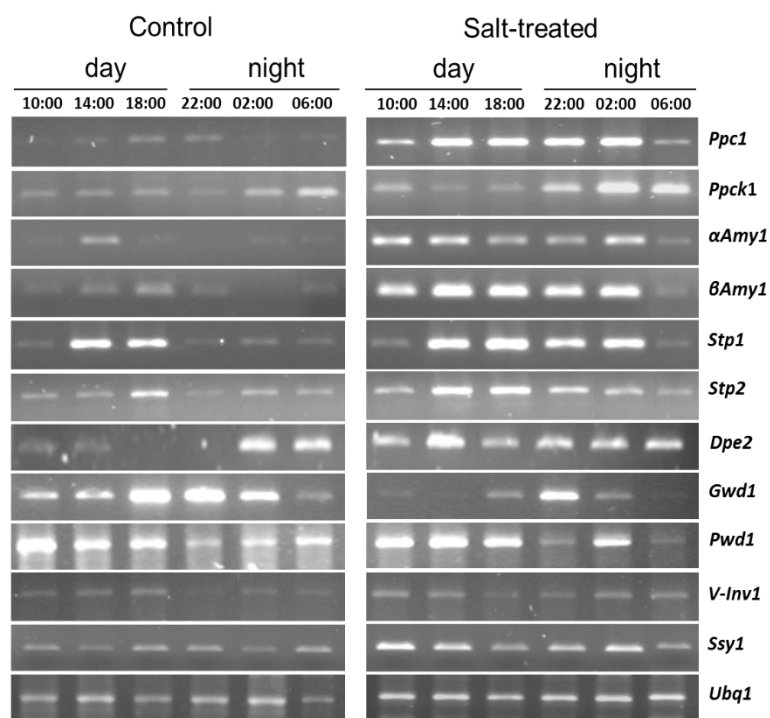


Figure 2.

A



B

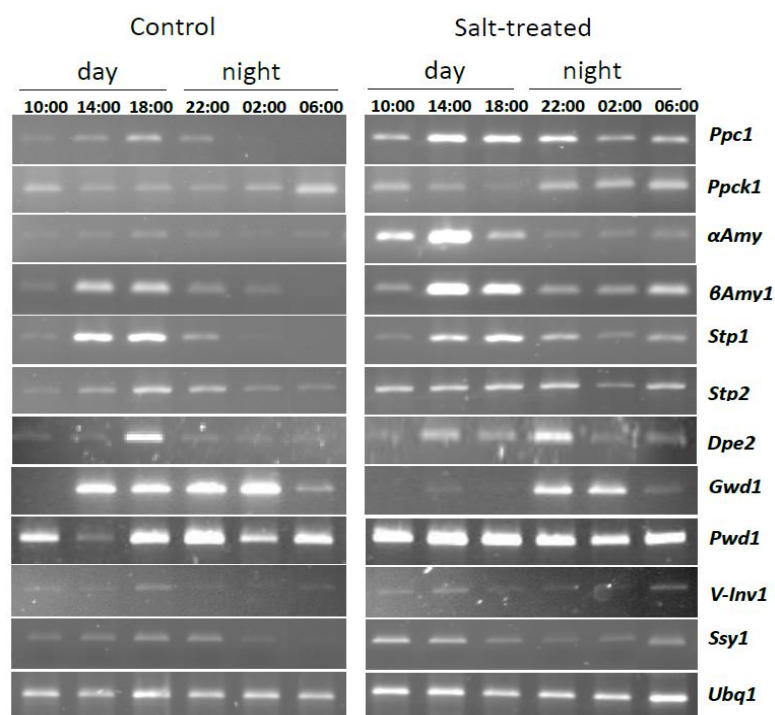


Figure 3.

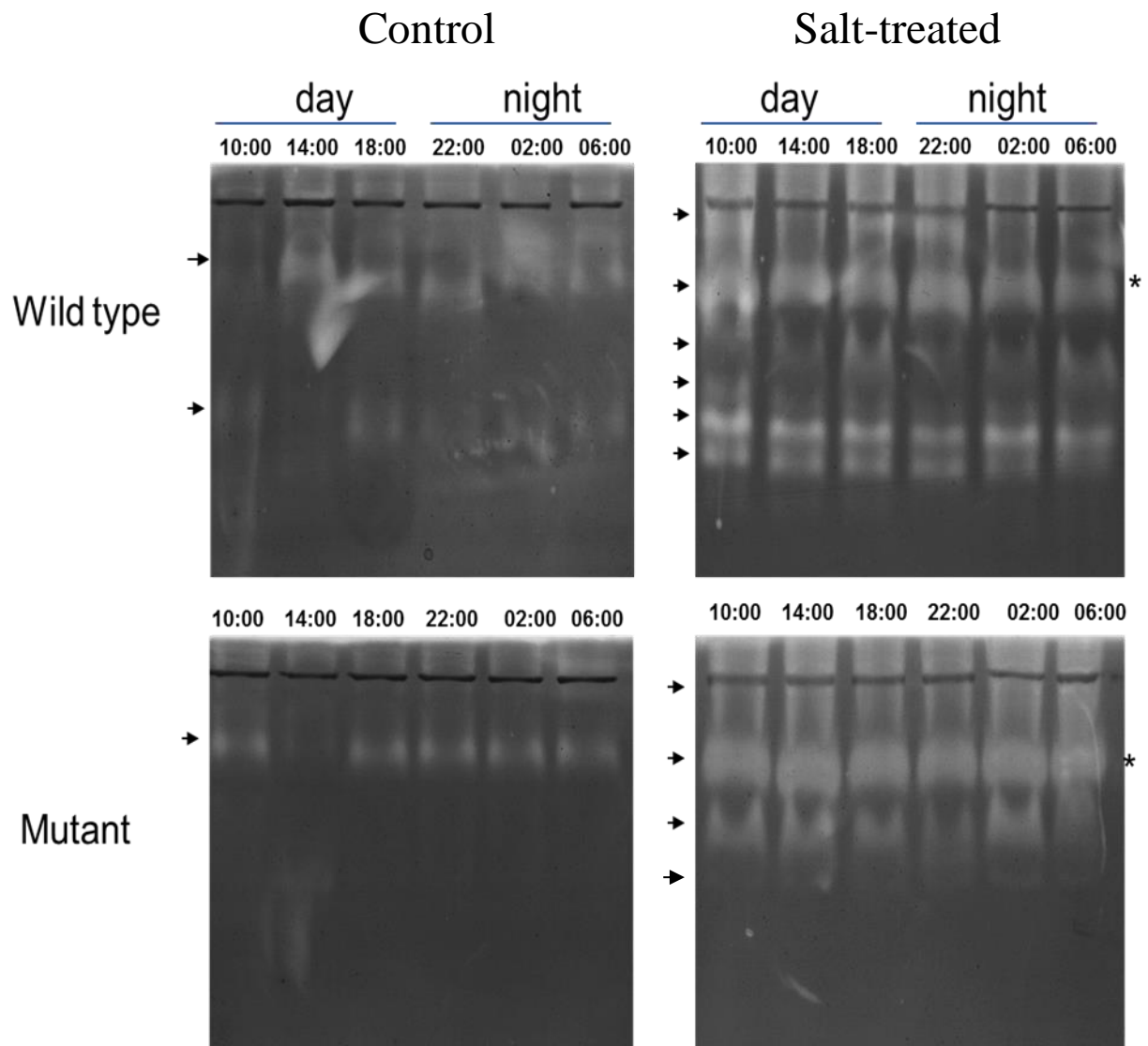


Figure 4.

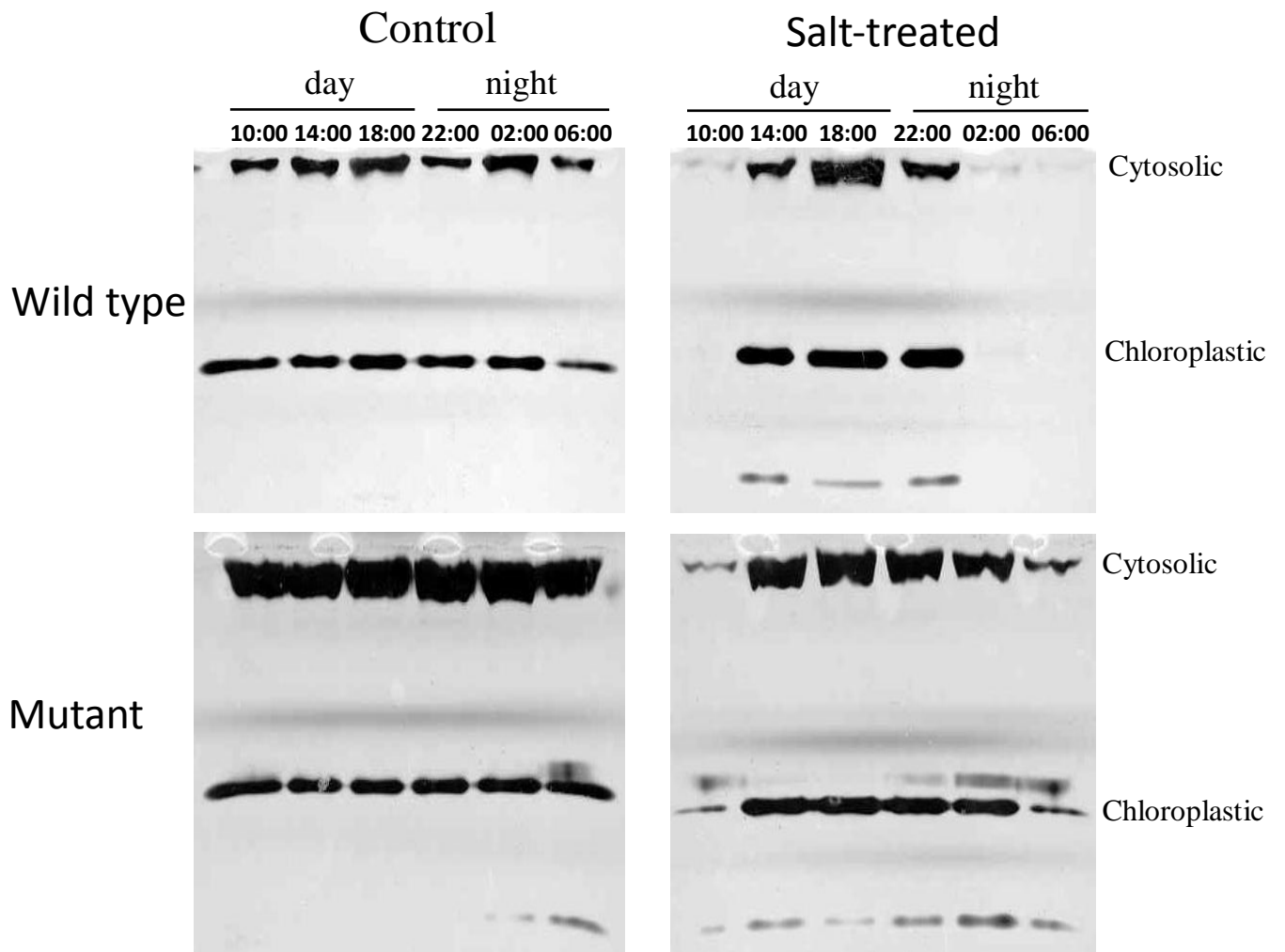


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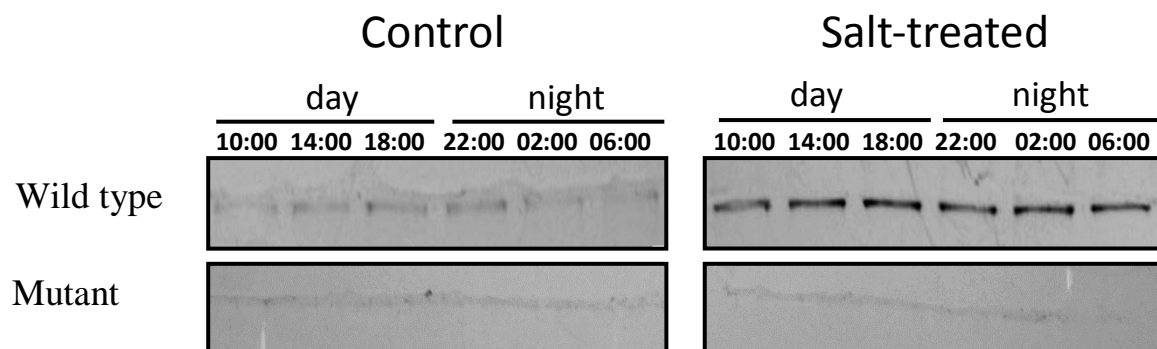


Figure 6.

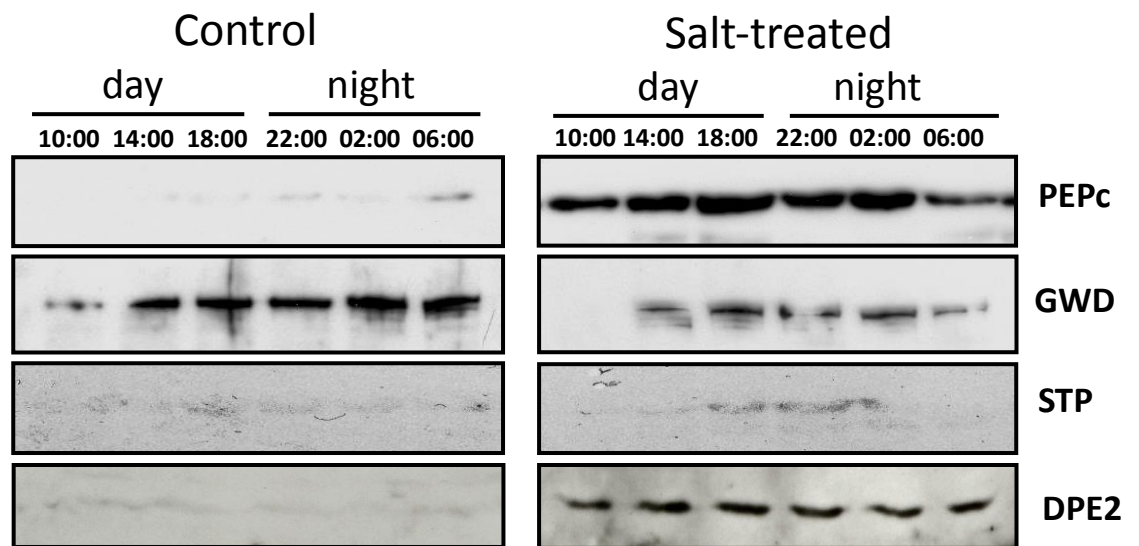
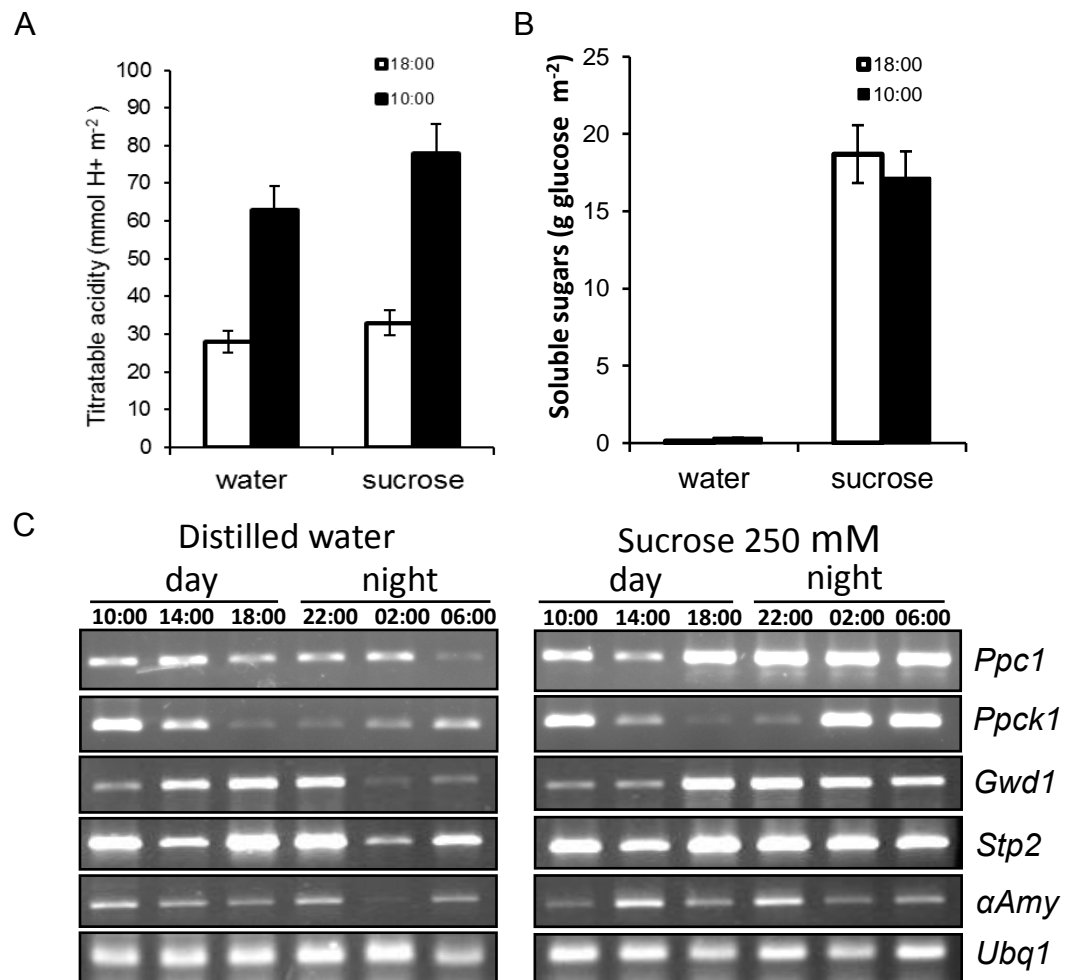
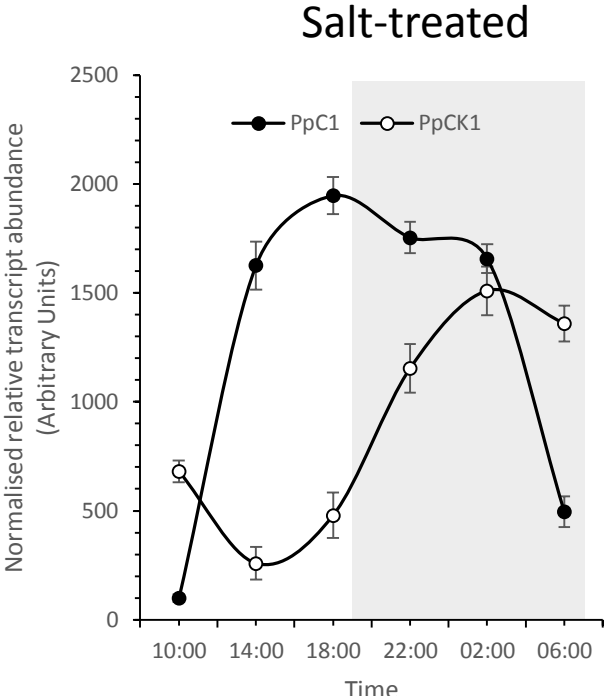
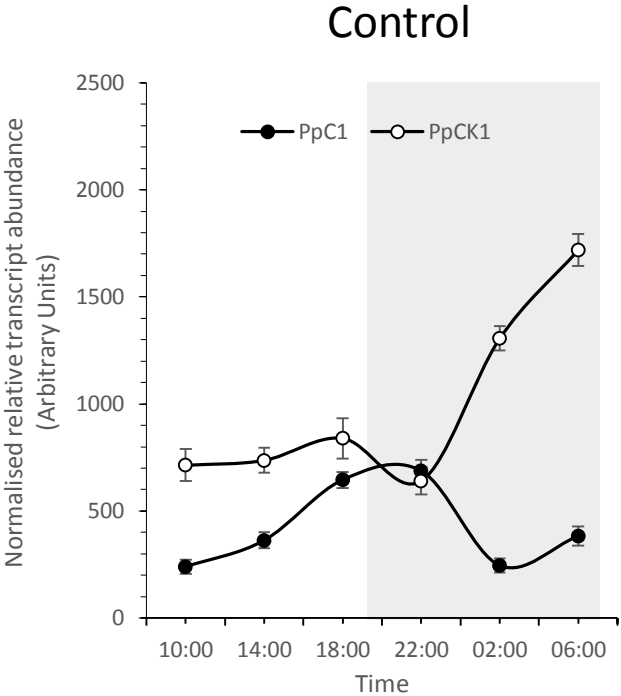


Figure 7.

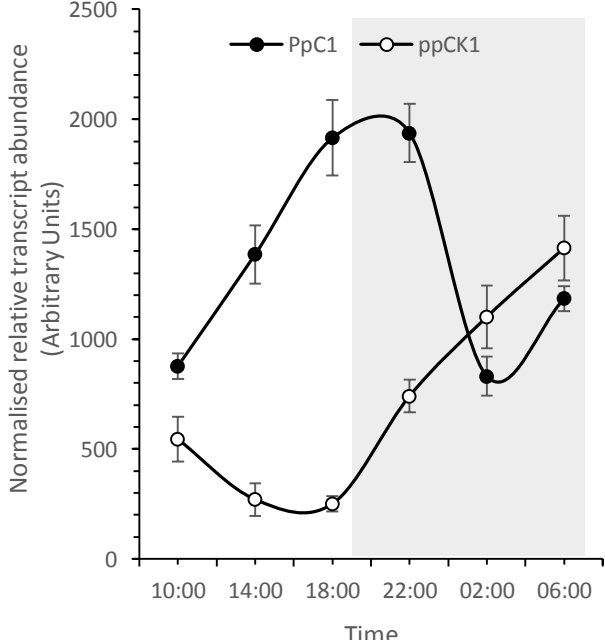
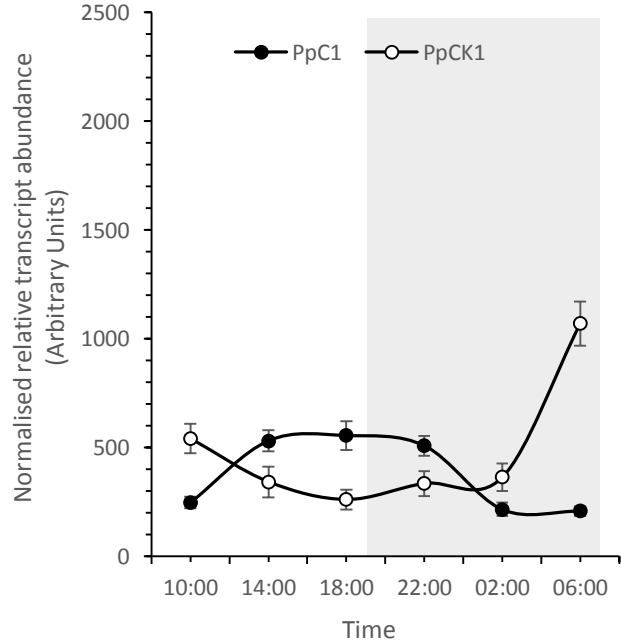


Supplementary figure S1

Wild type

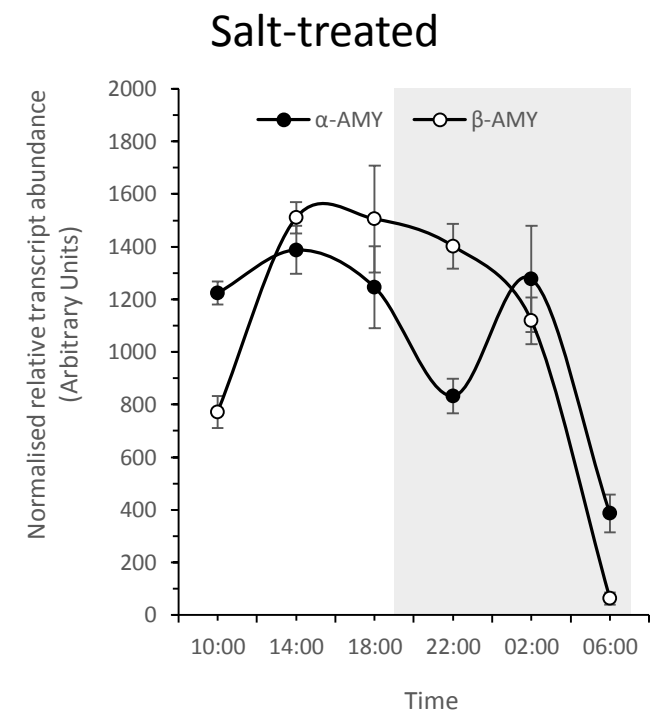
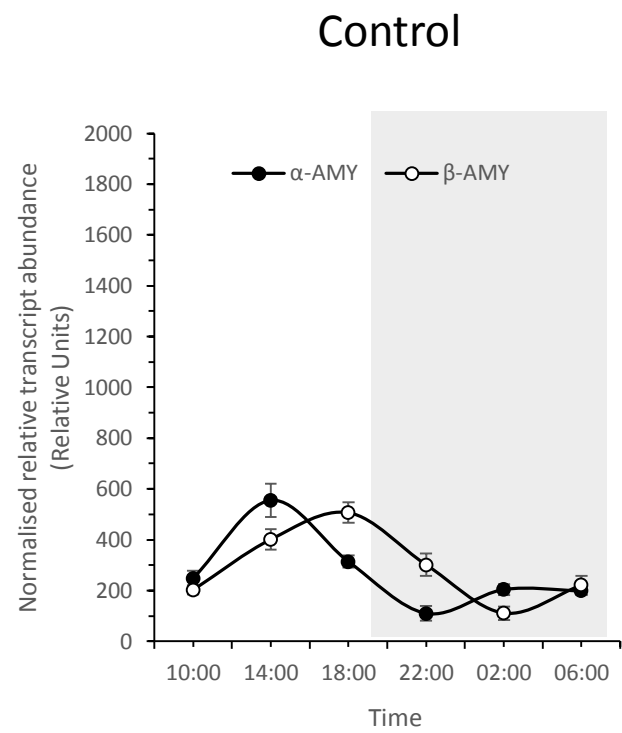


Mutant

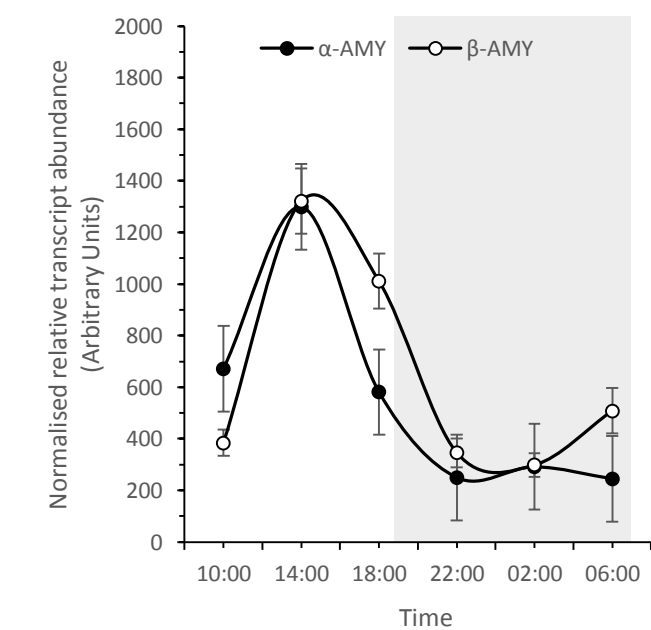
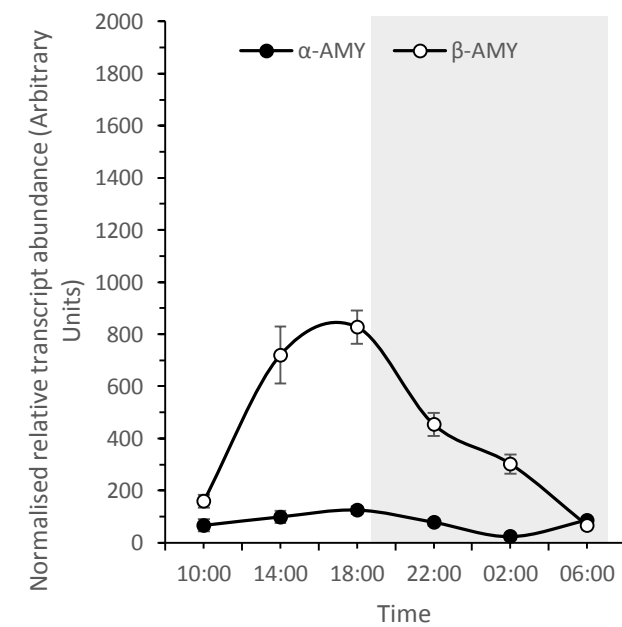


Supplementary figure 2

Wild type

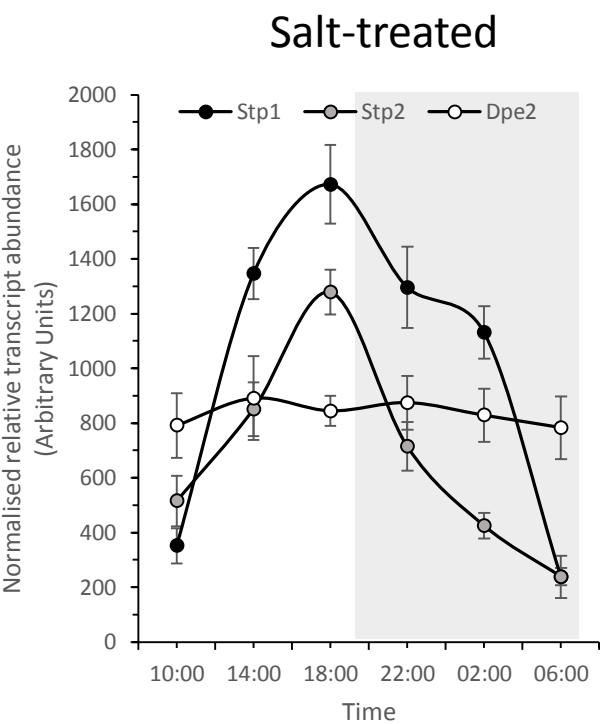
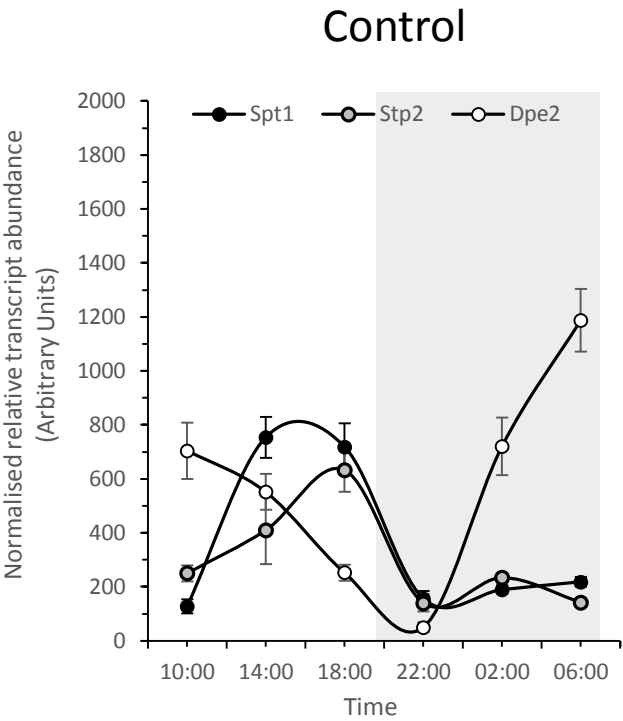


Mutant

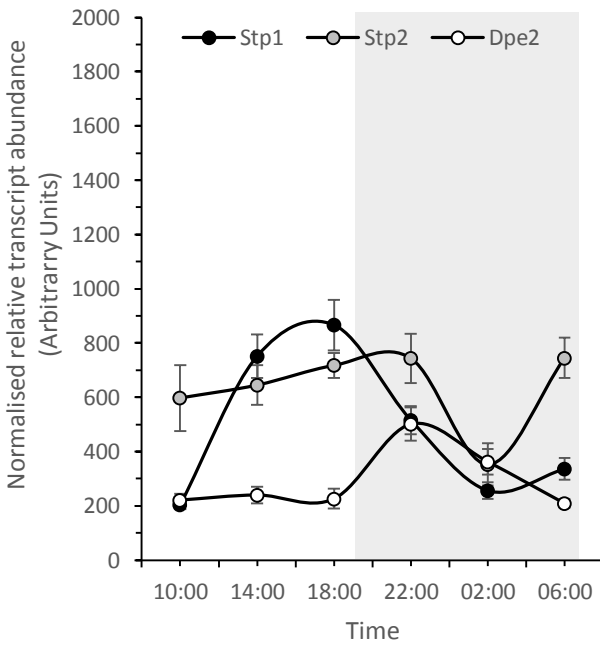
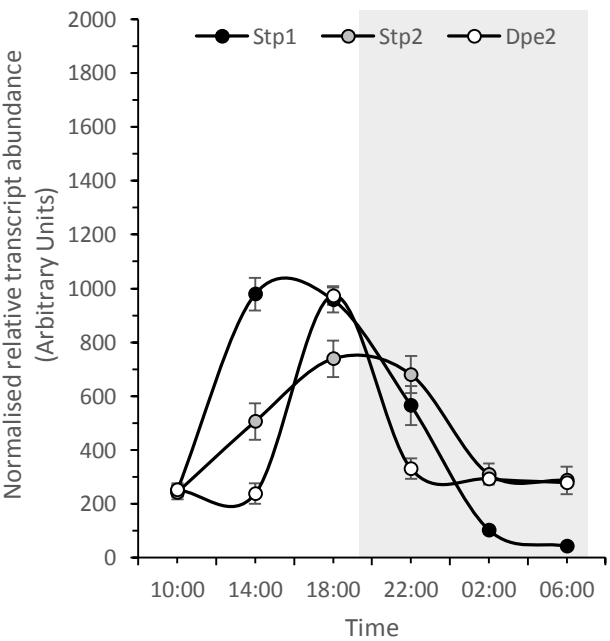


Supplementary figure 3

Wild type

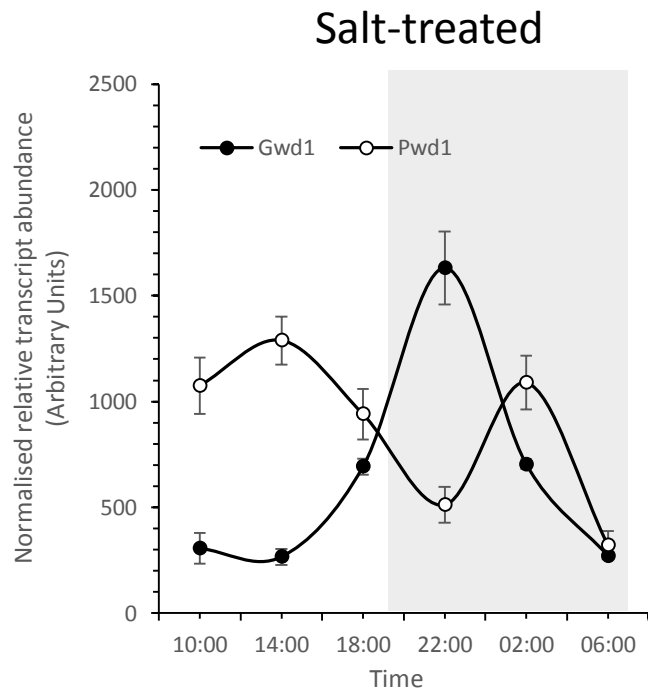
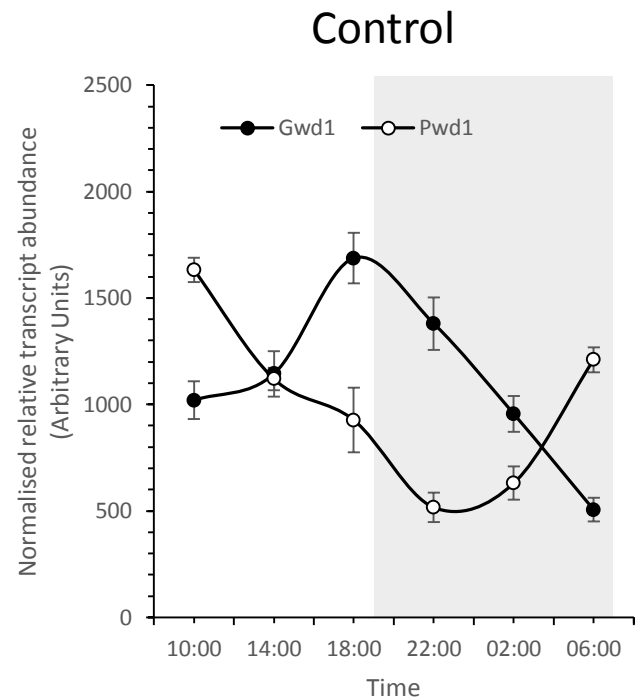


Mutant

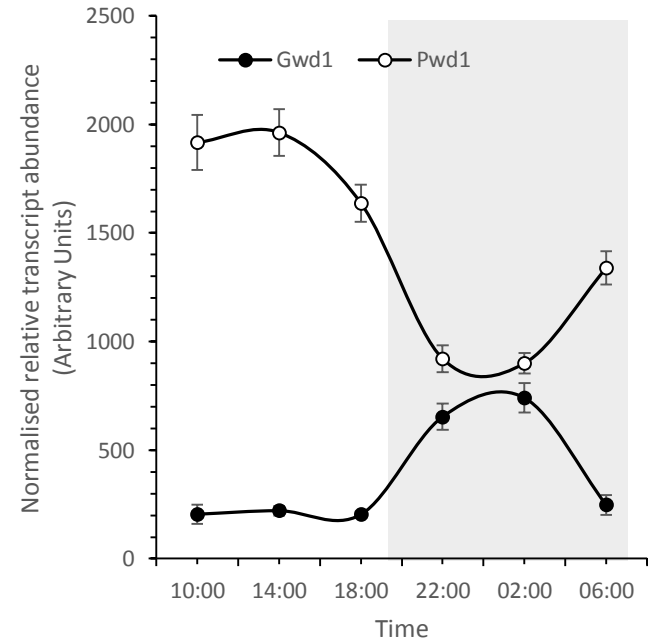
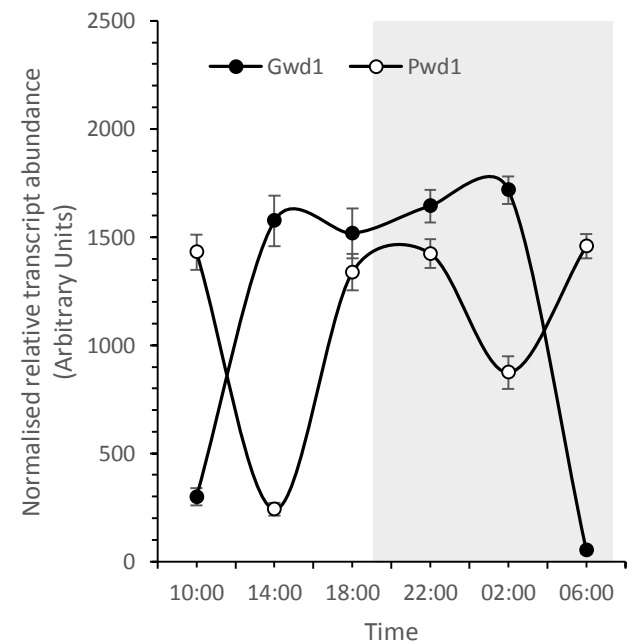


Supplementary figure 4

Wild type

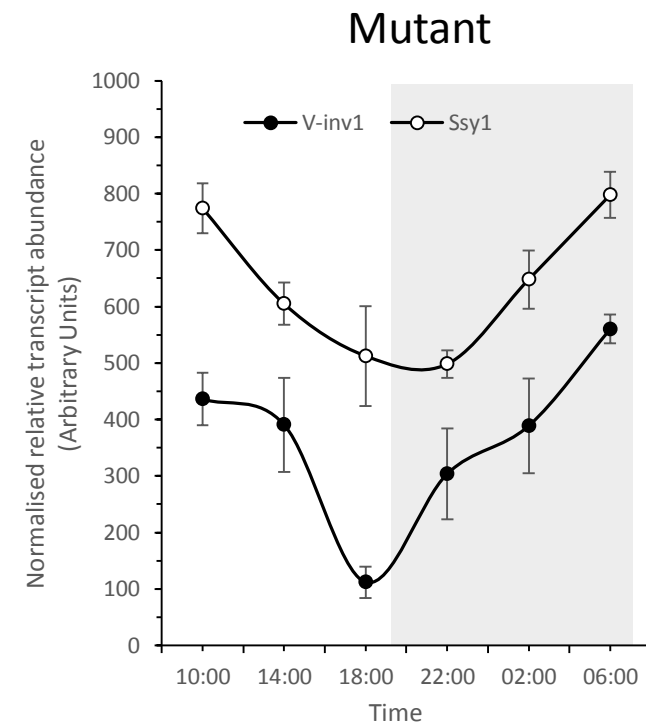
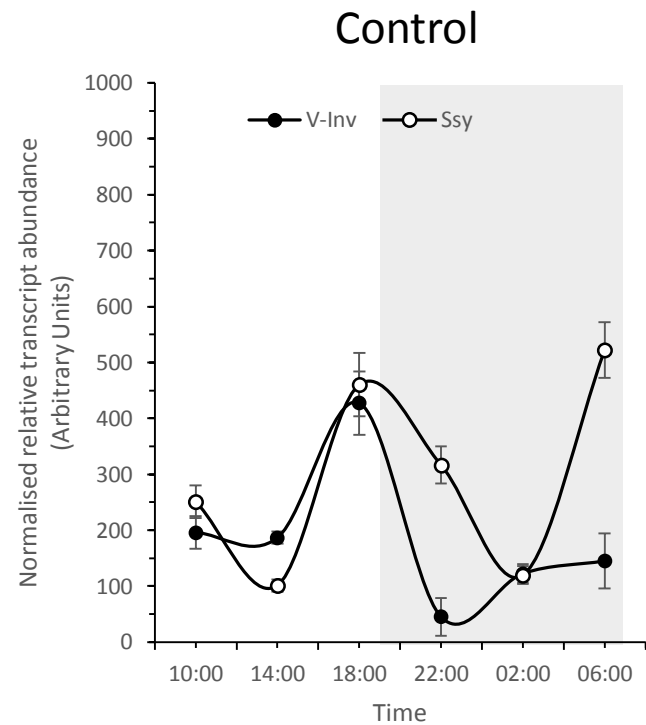


Mutant



Supplementary figure 5

Wild type



Mutant

